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Original Article

Influence of GnRH Agonist and Neural Antagonists on Stress-blockade of LH and Prolactin Surges Induced by 17β-estradiol in Ovariectomized Rats

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In our previous study, we demonstrated that immobilization stress blocked estrogen-induced luteinizing hormone (LH) surge possibly by inhibiting the synthesis and release of gonadotropin-releasing hormone (GnRH) at the hypothalamic level and by blocking estrogen-induced prolactin (PRL) surge by increasing the synthesis of dopamine receptor at the pituitary level in ovariectomized rats.

The present study was performed to determine whether immobilization stress affects pituitary LH responsiveness to GnRH, and whether endogenous opioid peptide (EOP) and dopamine systems are involved in blocking LH and PRL surges during immobilization stress. Immobilization stress was found to inhibit basal LH release and to completely abolish LH surge. However, the intravenous application of GnRH agonist completely restored immobilization-blocked LH surge and basal LH release. Treatment with naloxone did not exert any effect on immobilization-blocked LH surge but increased basal LH release during immobilization stress. Pimozide did not affect immobilization-blocked LH surge or basal LH release. Naloxone also decreased immobilization-induced basal PRL release, but had no effect on immobilization-blocked PRL surge. Immobilization-increased basal PRL levels were augmented by pimozide treatment and immobilization-blocked PRL surge was dramatically restored by pimozide.

We conclude that immobilization stress does not impair pituitary LH response to GnRH, and that the immobilization stress-induced blockage of LH surge is probably not mediated by either the opioidergic or the dopaminergic system. However, immobilization-blockade of PRL surge may be partly mediated by the dopaminergic system.

Key Words: Stress, LH, prolactin, immobilization, gonadotropin-releasing hormone, dopamine, opioid peptides

INTRODUCTION

It is well known that stress interferes with the reproductive function in human and animals.[1-3] A large amount of work has been devoted to understanding the mechanisms by which stress inhibits reproductive function. The activation of the hypothalamic-pituitary-adrenal (HPA) axis during stress is known to affect the hypothalamic-pituitary-gonadal (HPG) axis, especially by modulating the secretion and pulse frequency of gonadotropin.[4]

In our previous study, we found that immobilization stress inhibited estrogen-induced surges of luteinizing hormone (LH) and prolactin (PRL) in ovariectomized rats.[5] Furthermore, this stress decreased gonadotropin-releasing hormone (GnRH) mRNA levels in the preoptic area (POA) of the hypothalamus and increased GnRH content in the mediobasal hypothalamus, suggesting that immobilization stress may block LH surge by inhibiting the synthesis of GnRH in the POA and GnRH release from the median eminence at the hypothalamic level.[5] At the pituitary level, GnRH receptor mRNA levels were decreased during immobilization stress,[5] suggesting the possibility that such stress might decrease the pituitary's

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sensitivity to GnRH.

The question remains as to how immobilization stress modulates GnRH neuronal activity, which seems to be influenced by various neurotransmitters and neuropeptides.[6] Since evidence has indicated that endogenous opioid peptides (EOP) exert a tonic inhibitory role in LH secretion by inhibiting hypothalamic GnRH release,[7,8] there is a possibility that the stress-blockade of the LH surge is mediated by the EOP system.

Dopamine is a catecholamine that is released during stress.[9] Although no strong evidence exists that supports the direct effect of dopamine on the HPG axis, it seems to participate by inhibiting GnRH release indirectly. A profound increase in dopaminergic tone may also stimulate corticotropin-releasing hormone (CRH) release, which would be followed by an increase in EOP release,[10] and this in turn would suppress GnRH release.

The mechanism by which stress blocks PRL surge has not been determined. Tuberoinfundibular dopaminergic (TIDA) neuron is well accepted as a major physiological inhibitor of PRL secretion from the anterior pituitary,[11] and TIDA neuronal activity is attenuated during the prolactin surge period, suggesting that a decrease in DA secretion is responsible for the afternoon PRL surge.[12] In our previous study,[5] it was demonstrated that dopamine D2 receptor mRNA levels were decreased at the time of the PRL surge, but immobilization stress blocked the lowering of D2 receptor mRNA levels, suggesting that the dopaminergic system might play a role in the blockade of the PRL surge via the D2 receptor during immobilization.

The purpose of present study was, therefore, to determine whether immobilization stress decreases pituitary LH responsiveness to GnRH and to demonstrate whether the EOP and dopamine systems are involved in blocking the LH and the PRL surges during immobilization stress.

MATERIALS AND METHODS

Animals and experimental design

The following procedure was reviewed by the Committee for the Care and Use of Laboratory Animals at Yonsei University, according to the Guidelines and Regulations for the Use and Care of Animals at Yonsei University. Adult female Sprague-Dawley rats (250 - 320 g, Medical Research Center at Yonsei University College of Medicine) were housed in temperature (22℃)- and humidity (55%)-controlled conditions under a 12 hr light 12 hr dark photocycle (light on at 06.00 h), and food and water were supplied *ad libitum*. All were bilaterally ovariectomized (OVX) under light ether anesthesia and animals were used two weeks later. The number of animals in each group varied between 5 and 8.

Experiment 1: Two days before the experiment, a silastic capsule (30 mm in length, id 1.575 mm, od 3.175 mm; Dow Corning, Silastic Medical Grade Tubing) containing 17β-estradiol (E2, 180 μg/ml in sesame oil) or vehicle was implanted subcutaneously near the necks of OVX rats at 10.00 h, and a catheter was implanted in the right jugular vein for blood sampling. On the day of the experiment, 1 h before the beginning of blood sampling, the rats were randomly divided into two groups, i.e., non-stressed (control) and stressed groups. Immobilization stress was applied by placing the animals in an acryl restrainer (purchased from Myungjin Co., Seoul, Korea) from 10.00 h to 12.00 h for 2hours. In the case of the control group, some animals were allowed to move freely. Blood samples (0.3 ml) were taken from the jugular vein catheter at 15-min intervals from 10.00 h, and this was replaced with the same volume of heparinized saline at each sampling. Immediately after the third bleeding at 10.30 h, one of the test chemicals or saline was injected intravenously through the sampling catheter.

Experiment 2: This experiment was designed to examine the effects of GnRH agonist, naloxone and pimozide on stress-blocked E2-induced LH and PRL surges in OVX rats. E2 was administered to OVX rats and a catheter was inserted into the jugular vein, according to the same method described in the first experiment. Immobilization stress was applied from 13.00 to 21.00 h. Immobilization stress was not applied to the control animals. At 16.00 h, chemical or saline was administered intravenously. Blood samples

were taken at 30-min or 1-h intervals from 13.00 h.

Chemicals

Des-Gly10,[D-Ala6]-LH-RH ethylamide (Sigma, St Louis, MO., USA; 20 ng/kg) as a GnRH agonist, naloxone hydrochloride (Sigma; 2 mg/kg) or pimozide (Tocris Cookson Inc., Ballwin, Mo., USA; 1 mg/kg) was dissolved in physiological saline and injected at 1 ml/kg. Rats serving as controls were injected with the same volume of physiological saline.

Radioimmunoassay for LH and PRL

Blood samples were immediately centrifuged at 4,000 × g for 10 min, and the plasma was frozen at -20℃ until use. LH and PRL levels were assayed using double antibody radioimmunoassay reagents kindly provided by the National Hormone and Pituitary Program, NIDDK. NIDDK-rLH-I-9 and NIDDK-rPRL-I-6 were radioiodinated by the chloramine-T method. Antisera were prepared with NIDDK-anti-rLH-S-10 and NIDDK-anti-rPRL-S-9, and the reference preparations used were NIDDK-rLH-RP-2 and NIDDK-rPRL-RP-3 for LH and PRL, respectively. The intra- and inter-assay coefficients of variation of RIA were 5 - 7% and 8 - 10%, respectively.

Statistics

The values of the areas under the curves (AUC) for LH surge were compared between groups using one-way analysis of variance and the Student's t test. The AUC value was calculated from the plots in figure 1B according to the equation described by Akema et al.[13]:

$$AUC = \frac{1}{4}\sum_{n=1}^{4}[(X_{n-1}+X_n)/2 - X_0] + \frac{1}{3}\sum_{n=5}^{7}[(X_{n-1}+X_n)/2 - X_0]$$

[ng/ml × h],

where X_0 was the basal LH value at 16.00 h, X_1, X_2, ..., and X_4 were the hormone values at 16.30, 17.00, ..., and 18.00 h, and X_5, X_6, and X_7 the hormone values at 19.00, 20.00, and 21.00 h.

Plasma hormone values in the present study were statistically analyzed by analysis of variance followed by Duncan's test. P value of less than 0.05 was considered statistically significant.

RESULTS

Effect of immobilization stress on pituitary LH response to GnRH

To determine whether immobilization stress impairs pituitary sensitivity to GnRH, and thereby inhibits LH release during the non-surge and the surge period, the effect of GnRH agonist was evaluated on immobilization-blocked basal LH release and LH surge.

Treatment with GnRH agonist dramatically increased basal plasma LH levels in the non-stressed and the stressed groups in the morning. In the non-stressed group, GnRH agonist started to increase LH release within 15 minutes and this increase was continued thereafter. Although immobilization stress significantly lowered basal levels of plasma LH, treatment with GnRH agonist increased plasma LH levels in a similar manner to that observed in the non-stressed group (Fig. 1A).

As shown in figure 1B, E-induced LH surge in OVX rats began at about 16.00 h and reached a peak at 18.00 h. Treatment with GnRH agonist advanced surge and increased the amplitude of surge in the non-stressed rats. Interestingly, treatment with GnRH agonist restored LH surge which was blocked by immobilization stress. This result was confirmed by AUC values, which reflected the total amount of LH released within a given period. In the non-stressed rats, the AUC value was 14.9 ± 2.97 ng/ml(h during the surge period and treatment with GnRH agonist did not affect the mean AUC value (Fig. 2). In immobilized rats, the AUC value was significantly reduced to 0.6 ± 1.2 ng/ml·h. However, treatment with GnRH agonist fully restored the reduced AUC value to the control level (14.3 ± 2.6 ng/ml·h).

Effect of naloxone on LH release under immobilization stress

Fig. 3A shows that during the non-surge period, treatment with naloxone, an opiate antagonist,

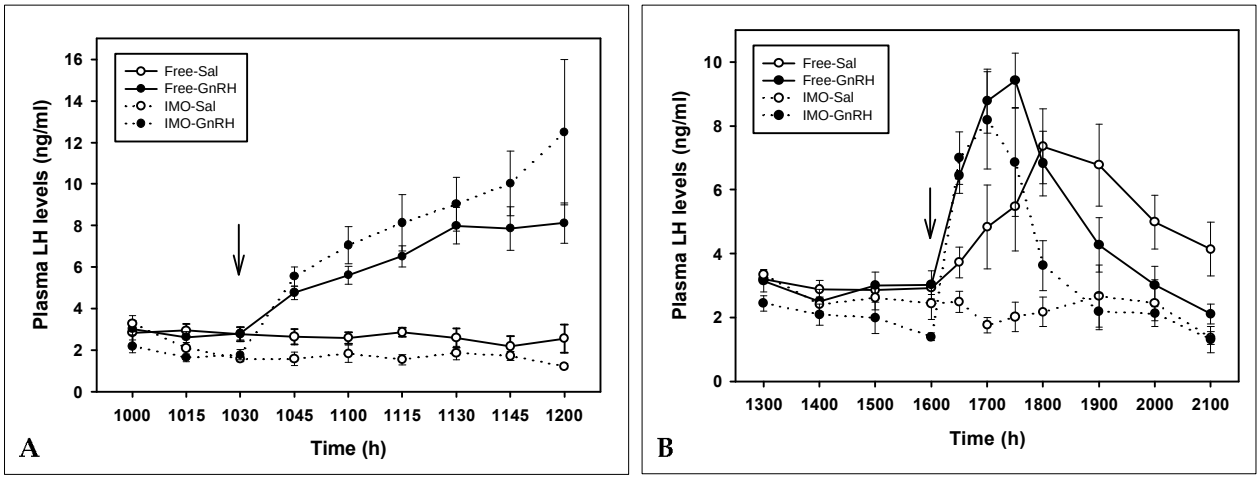

Fig. 1. Effect of GnRH agonist treatment (20 ng/kg) on plasma LH levels during the non-surge period (A) and the surge period (B). GnRH agonist was injected intravenously through a sampling catheter at 10.30 h (A) and 16.00 h (B). Data points represent means ± SE (n=5-8). Sal, saline; GnRH, gonadotropin-releasing hormone; IMO, immobilization.

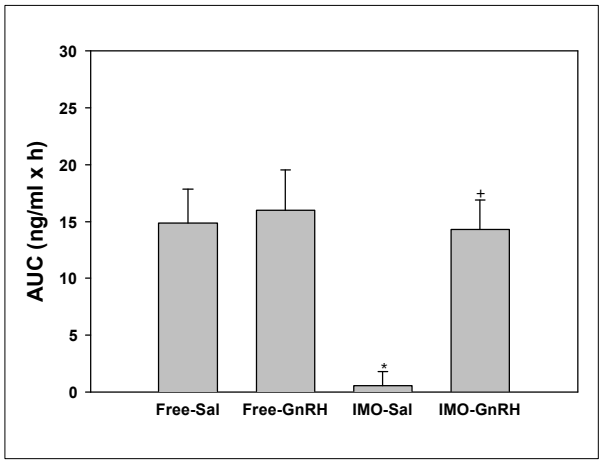

Fig. 2. Areas under the curve (AUC) above basal LH after GnRH treatment (20 ng/kg) in free and immobilized rats during the surge period. Each bar represents the mean ± SE. *$p<0.001$ vs. Free-Sal, + $p<0.001$ vs. IMO-Sal. Abbreviations: see legend of fig. 1.

dramatically increased plasma LH levels in both non-stressed and stressed rats, reaching the maximum within 30 min after naloxone injection. In contrast to the non-surge period, treatment with naloxone did not exert any significant effect on LH surge. In the non-stressed rats, LH surge appeared to be advanced by 30 min by naloxone treatment but this advance was not statistically significant. Immobilization-blocked LH surge was not restored by naloxone treatment (Fig. 3B).

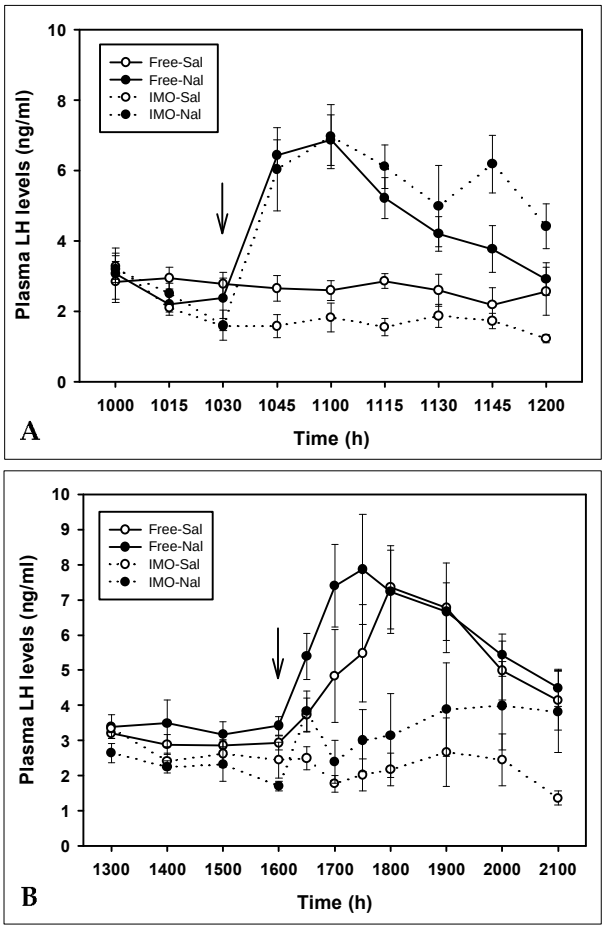

Fig. 3. Effect of naloxone treatment (2 mg/kg) on plasma LH levels during the non-surge period (A) and the surge period (B). Naloxone was injected intravenously through a sampling catheter at 10.30 h (A) and 16.00 h (B). Data points represent means ± SE (n=5 - 7). Abbreviation: Nal, naloxone.

Effect of pimozide on LH release under immobilization stress

During the non-surge period, treatment with pimozide (1 mg/kg), a dopamine D2-receptor antagonist, failed to modulate plasma LH levels in the stressed or in the non-stressed rats (Fig. 4A). The LH surge was not significantly affected by pimozide treatment although the surge was blunted slightly (Fig. 4B). Immobilization-blocked LH surge was not modulated by pimozide treatment (Fig. 4B).

Effect of naloxone on prolactin release under immobilization stress

During the non-surge period, immobilization stress induced acute PRL release and plasma PRL levels remained significantly elevated (Fig. 5A). Treatment with naloxone acutely decreased PRL levels in immobilized rats ($p<0.001$). During the surge period, immobilization stress blocked the E-induced PRL surge. Naloxone treatment did not affect the immobilization-blocked PRL surge (Fig. 5B).

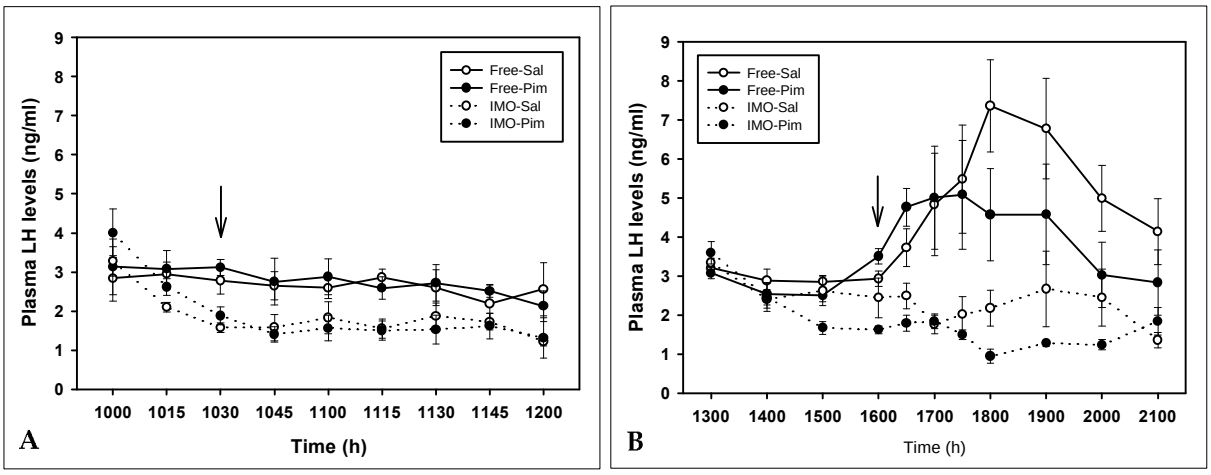

Fig. 4. Effect of pimozide treatment (1 mg/kg) on plasma LH levels during the non-surge period (A) and the surge period (B). Pimozide was injected intravenously through a sampling catheter at 10.30 h (A) and 16.00 h (B). Data points represent means ± SE (n=5 - 8). Abbreviation: Pim, pimozide.

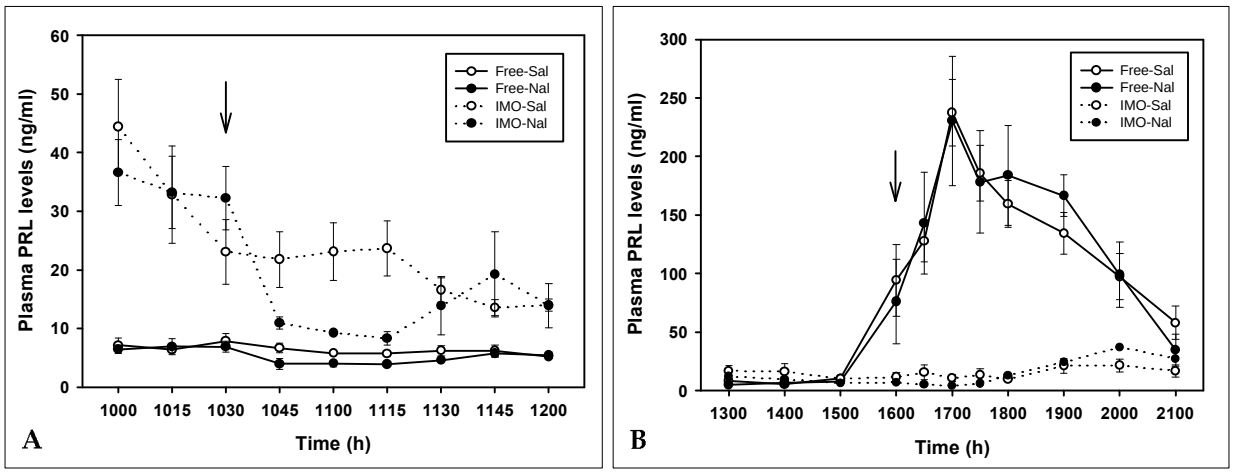

Fig. 5. Effect of naloxone treatment (2 mg/kg) on plasma PRL levels during the non-surge period (A) and the surge period (B). Naloxone was injected intravenously through a sampling catheter at 10.30 h (A) and 16.00 h (B). Data points represent means ± SE (n=5 - 7). Abbreviations: see legend of fig. 3.

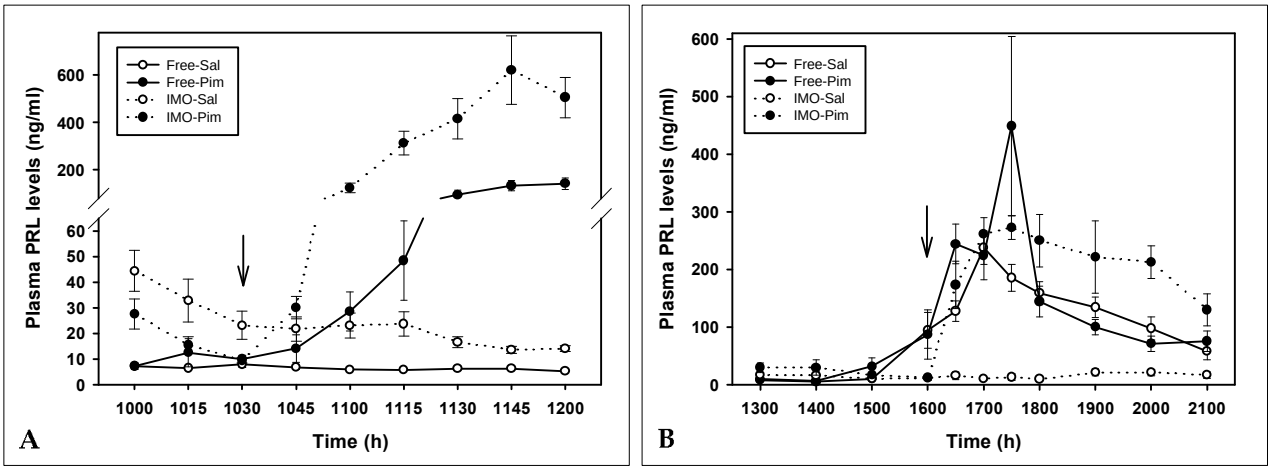

Fig. 6. Effect of pimozide treatment (1 mg/kg) on plasma LH levels during the non-surge period (A) and the surge period (B). Pimozide was injected intravenously through a sampling catheter at 10.30 h (A) and 16.00 h (B). Data points represent means ± SE (n=5 - 8). Abbreviations: see legend of fig. 4.

Effect of pimozide on PRL release under immobilization stress

During the non-surge period, pimozide treatment dramatically increased PRL release. In the stressed group, plasma PRL levels were augmented by pimozide treatment as compared to the non-stressed group (Fig. 6A). Pimozide treatment completely restored the immobilization-abolished PRL surge (Fig. 6 B).

DISCUSSION

The present study demonstrates that immobilization stress does not impair the pituitary LH response to GnRH. Treatment with GnRH agonist completely restored stress-blocked LH release during the non-surge and the surge periods. This result is supported by an earlier report that chronic stress (8 h/day for 10 days) decreased plasma LH levels but did not affect LH synthesis or responsiveness to GnRH in the pituitary.[14] In our previous report,[5] LHβ mRNA levels determined during the surge were unaffected by immobilization. On the other hand, the GnRH content of the mediobasal hypothalamus during the surge was increased by immobilization stress, whereas GnRH mRNA levels were decreased in the POA. Thus, our results strongly suggest that immobilization stress does not impair pituitary responsiveness to GnRH, but rather blocks LH surge by inhibiting synthesis and release of GnRH at the hypothalamic level.

During the non-surge period, naloxone treatment rapidly increased plasma LH levels in the non-stressed rats. It has been suggested that endogenous opioids exert a tonic inhibition on pulsatile LH secretion, and may mediate the negative feedback action of estrogen on pulsatile LH secretion during the non-surge period.[7] On the other hand, decreases in β-endorphin levels of the hypophyseal portal blood, in hypothalamic β-endorphin stores,[15,16] in pro-opiomelanocortin (POMC) mRNA, and in POMC primary transcript[17-19] have been found in the afternoon of proestrus. These results imply that reduced β- endorphin influence is required for preovulatory LH surge. The finding that inhibition of LH secretion by CRH administration was blocked by an administration of naloxone implies that CRH inhibits LH release through an EOP mechanism.[20] Furthermore, the *in vivo* and *in vitro* release of β-endorphin and dynorphin from the hypothalamus was stimulated by CRH, which suggests that the inhibitory action of stress on LH release is partly, mediated by the EOP system. In the present study, naloxone treatment induced an abrupt elevation in plasma LH levels in the control and the stressed group without inducing a significant difference during the non-surge period when the tonic action of EOP prevails. However, during the

surge period, when EOP activity is low, naloxone treatment did not have a significant effect on stress-blocked LH surge. Therefore, our results suggest that the blockade of LH release during immobilization may not be mediated effectively by the EOP system.

The role of dopaminergic neurons in regulating LH secretion seems to be controversial. It has been reported that dopamine exerts an inhibitory effect on LH secretion, possibly by inhibiting GnRH release,[21,22] whereas dopamine was found to have a stimulatory effect on LH release.[23] In the present study, pimozide treatment did not modulate basal LH release and LH surge in the non-stressed or in the stressed rats. These results are consistent with earlier reports that the blockade of DA receptors with specific antagonists showed no effect on the basal levels of LH.[24,25] Furthermore, immobilization-blocked LH release was not ameliorated by pimozide treatment in the present study. Thus, immobilization stress does not seem to modulate dopaminergic activity to inhibit LH release, at least in the present study. However, there is a report that the nicotine-induced inhibition of LH secretion was blocked by the D1 receptor antagonist and not by the D2 receptor antagonist,[26] suggesting that dopamine cannot be entirely ruled out. Pimozide, used in the present study was found to have a higher selectivity for the D2 receptor than the D1 receptor. To clarify this issue further study should be performed with specific antagonists for the dopamine receptor.

Studies using a wide variety of opiate antagonists indicate that endogenous opioids indeed play an important role in regulating PRL secretion.[27-29] Although naloxone alone induced a slight decrease in the basal circulating PRL level, it was quite effective at lowering PRL levels stimulated during suckling or stress.[30,31] The present study shows that naloxone administration attenuates PRL-hypersecretion induced by immobilization during the non-surge period. Thus, the present results with those of others confirm that EOPs might play a role in the stress-induced release of basal PRL during the non-surge period. During the surge period, however, naloxone treatment failed to modulate PRL surge in both non-stressed and stressed rats.

It is well known that dopamine is a potent inhibitor of PRL secretion.[32] The present study shows that pimozide induces a dramatic increase in PRL levels during the non-surge period. It is interesting that the stressed animals proved to be more responsive to pimozide, which suggests that additional factors are induced when the animals are under stress and that these are more active when dopaminergic signals are inhibited. Dermarest et al.[33] reported that acute restraint stress decreases tuberoinfundibular dopaminergic neuronal activity, and Shin et al.[34,35] suggested that stress triggers PRL release by activating putative PRL-releasing factors. Moreover, some putative PRL-releasing factors, such as serotonin,[36] histamine[37,38] and vasopressin[37] may be activated by pimozide and induce PRL release under stress. Further studies are required to confirm this hypothesis.

The mechanism by which stress blocks PRL surge has not been clearly elucidated. The present study shows that immobilization suppresses proestrus-like E2-induced PRL surge and that treatment with pimozide restores this blockade, suggesting that the blockade of PRL surge by the immobilization stress may be mediated by the dopaminergic system. It is known that the suppressing effect of restraint stress on the afternoon- and nocturnal PRL surges is accompanied by an increase in TIDA neuronal activity.[12,39] However, since passive immunoneutralization of endogenous TRH[40] or treatment with oxytocin antagonist[41] also inhibits the proestrus surge of PRL, the involvement of PRL-releasing factors in mediating the blockade of PRL surge by immobilization is possible. However, considering the present result that stress-blocked PRL surge is fully restored by pimozide treatment, increased dopaminergic activity, especially TIDA neuronal activity, seems to be a major component of the mechanism by which immobilization stress blocks PRL surge.

In conclusion, the present study strongly suggests that immobilization stress does not impair the pituitary LH response to GnRH. Immobilization stress-induced blockade of LH surge is not likely to be mediated by either the opioidergic or the dopaminergic systems. The opioidergic system also might not mediate the immobilization-induced inhibition of PRL surge, while the dopaminergic system is likely to play a key role in this event.

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